Antitumor activities and immunological studies with algal polysaccharides

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SUMMARY: The antitumor effect of G IV-A (fucoidan) and/or 5-FU was examined in an experimental model of lung metastases induced by Lewis lung carcinoma (3LL) or Ehrlich ascites carcinoma (EAC) in mice. Combination treatment with G IV-A and 5-FU increased significantly antitumor activity. In the group receiving G IV-A, the percentages of splenic Thy1.2-, L3T4- and asialo GM1-positive cells were significantly increased as compared with the tumor-bearing mice treated with saline. Furthermore, the L3T4⁺/Lyt2⁺ ratio showed a tendency to increase, and the Lyt2⁺/Thy1.2⁺ ratio was decreased. These results suggest that the antitumor effect of G IV-A may be correlated with the changing pattern of the Thy1.2-, L3T4- and asialo GM1-positive cells, the third component of complement C3 activation, and macrophage activation.

KEY WORDS: seaweed, fucoidan, immune response, antitumor activity

INTRODUCTION

Antitumor drugs with direct cytotoxic or cytostatic effects on tumor cells commonly have side effects as well such as gastrointestinal and bone-marrow disturbances or hepatic and blood toxicity. These drugs also suppress immune responses. On the contrary, recently biological response modifiers like antitumor drugs have been developed in Japan. These antitumor drugs are Krestin, or PSK obtained from mycelium of Kewaratake (*Coriolus versicolor*), Lentinan from fruiting bodies of Shiitake (*Lentinus edodes*) and Schizophyllan a product from the medium of Suehirotake (*Shizophyllum commune*).

Certain seaweeds have long been used in traditional Chinese herbal prescriptions for the treatment of malignant tumors. In 1974, Yamamoto *et al*^{1,2)} reported that *Sargassum fulvellum*, *S. kjellmanianum*, *Laminaria angustata*, and *L. angustata* var. *longissima*, markedly inhibited the growth of Sarcoma-180 implanted subcutaneously in mice, and each fraction was found to consist primarily of polysaccharide. On the other hand, Nakazawa *et al*³⁾ also demonstrated that a sulfonic acid group-rich fraction from *Sargassum horneri* showed antitumor activity against EAC in mice. Several sulphated polysaccharides have been used experimentally as antimetastatic agents.⁴⁻⁶⁾

Harada *et al*⁷⁾ previously reported that methanol extracts of 47 species of marine algae from Japan's coasts was biologically active *in vitro* selective antitumor activity against several tumor cell lines.

In 1975, we examined the effects of 12 kinds of marine algae from the *Sargassum* group on the growth of EAC in mice. In particular, strong antitumor activity was seen in *Sargassum thunbergii* (Japanese name "Umitoranoo"). After the pre-treatment of EtOH, the polysaccharides fraction were isolated from a hot-water extract of Umitoranoo by ethanol precipitation, ion-exchange

chromatography and gel-filtration. A total of 32 polysaccharides were tested antitumor activities against EAC.⁸⁾ Especially, G IV-A fraction was most effective. G IV-A is fucan sulfate (fucoidan) of molecular weight 19,000 containing 10.4% uronic acid, 1.6% protein and 28.2% fucosyl-OH ester sulfate, judging from analyses of IR- and ¹³C-NMR-spectra.⁹⁾

The present studies were undertaken to examine the antitumor effects of G IV-A on the growth of EAC, 3LL and the inhibition of lung metastasis. In addition, the phagocytosis of macrophages, the alterations of complement C3, and the influence of the splenic lymphocyte subsets was discussed.

MATERIALS AND METHODS

Animals

Five-week-old female C57BL/6CrSLC mice (SPF) and ICR/Slc mice were obtained from Shizuoka Laboratory Animal Center (Japan).

Tumor cells

EAC was initially supplied by the National Cancer Center Research Institute, Tokyo, Japan. 3LL cells were kindly provided by Dr. H. Nakanishi, M.D.¹⁰⁾ Aichi Cancer Center Institute (Japan).

Chemicals

G IV-A was prepared according to the methods previously reported for the purification.⁹⁾

Evaluation of antitumor activity

EAC cells were inoculated intraperitoneally (i.p.) into the mice. Treatment was initiated 24 hrs after tumor inoculation. For the 3LL system, the test

samples were injected i.p. three times a week, starting 24 hrs after tumor implantation. As for the lung metastases system, 3LL cells were implanted s.c. into one footpad of each mouse.¹¹⁾

The C3 cleavage product binding assay

Eight-week old female mice were injected i.p. with zymosan, GIV-A. The percentage of C3-positive fluorescent cells was calculated by counting of 200 adherent cells.¹²⁾

Analysis of splenic T-lymphocyte subsets

GIV-A was given i.p. at a daily dose of 20 mg/kg three times a week for 2 weeks. The suspension of spleen cells were added monoclonal antibody. The cell suspension was analyzed by flow cytometry.¹³⁾

RESULTS

EAC are considered to be rapid growing tumors. So we examined antitumor effect of G IV-A against EAC. In 20 mg/kg treated group, the growth of the tumor was suppressed in all of the mice examined and the complete regression of tumor was observed in 5 out of 8 mice (Table 1). To investigate the antitumor mechanism of G IV-A, EAC cells were obtained from the peritoneal cavity in mice. After the incubation, the cells were stained with FITC-labeled G IV-A and examined under a Fluorescence microscope. EAC cells have the receptor which specifically bind to G IV-A (data not shown). Therefore, continued ability to bind G IV-A on the EAC cell membranes could play an important role in the antitumor response. ATSO and Picibanil showed no antitumor activity. While 5-FU was markedly active, causing an increase in life-span of 65.6% as controls. However, the median survival time of mice treated with 5-FU was shorter than that of G IV-A.

Recently, lung cancer is increased significantly in Japan. Then we examined the antitumor effect of GIV-A and 5-FU on 3LL. In the group receiving G IV-A 20 mg/kg, marked suppression of tumor growth was seen (Table 2). The tumor inhibition ratio of the GIV-A treated group relative to the saline-treated control group was 81.0%. Antitumor activity of GIV-A varied according to the dose. 5-FU 30mg/kg was markedly active. However, the antitumor effect of 50 mg/kg with 5-FU was weaker than that of the control group because of 5-FU toxicity.

The inhibition and prevention of metastasis are important problems in cancer therapy. We used 3LL as an experimental model. An optimal dose of G IV-A 20mg/kg and 5-FU 30 mg/kg was administered i.p.. As shown in Table 3, the development of lung metastases was inhibited. Combination treatment with G IV-A plus 5-FU significantly decreased the mean number of

Table 1. Antitumor effects of some preparations on EAC

Treatment	Mean survival time		Tumor-free mice on day 30
	Days	%	
Saline	15.1 ± 1.5	100	0/10
G IV-A	52.6 ± 11.3 *	> 348.3	5/ 8
ATSO	15.7 ± 2.3	103.9	0/10
Picibanil	18.3 ± 1.9	121.2	0/10
5-FU	25.0 ± 4.1 *	> 165.6	1/10

G IV-A (20 mg/kg), ATSO (100 mg/kg), picibanil (2 KE/kg) or 5-FU (20 mg/kg) on days 1 to 10. Survival time of the treated group expressed as a percentage of survival time of the controls. Values indicate the mean ± S.E. of 8 to 10 mice. * P < 0.05 compared with control group.

Table 2. Antitumor effects of G IV-A and 5-FU on 3LL

Treatment	Dose (mg/kg/day)	Average weight of tumor (g)	Tumor inhibition ratio (%)
G IV-A	10	4.3 ± 2.1	25.9
	20	1.1 ± 0.7 *	81.0
	50	2.6 ± 1.7 *	55.2
5-FU	10	4.1 ± 2.2	29.3
	30	1.6 ± 0.9 *	72.4
	50	Toxic	
Control		5.8 ± 2.0	0

Values are indicated as means ± S.E. of 9 mice. Significant difference: * P < 0.05, as compared with the control group.

metastatic nodules visible in the lung. No metastatic nodules were seen in 4 of the 10 mice treated with GIV-A plus 5-FU.

The infectious microorganism can be removed about 99% by epithelial surface membranes, complement, macrophages and leucocytes, cytokines, as humoral immunity. We examined the effect of G IV-A on human complement system. The C3 conversion activity of human serum treated with G IV-A was assayed by crossed immunoelectrophoresis. We found that the antitumor activities of ATSO, Zymosan and G IV-A is mediated through the activation of complement C3 by the alternative pathway.

Next, we examined the effect of G IV-A on macrophages (Table 4). The C3-positive macrophages in the peritoneal cavity were observed 2 hours after i.p.. The 67.5% macrophages were most brightly immunofluorescent positive cells by G IV-A. The enhanced phagocytosis is probably related to C3 activation by G IV-A.

Activated adherent peritoneal exudate cells were collected from mice after intravenously injection of G IV-A. Activated-macrophages significantly reduced the number of lung metastatic nodules (data not shown). These results indicate that activated-macrophages alone inhibited the development of lung metastasis. Macrophages are found in the reticuloendothelial organs such as liver, spleen, thymus, lymphoid tissue and bone marrow.

Table 3. Antitumor effects of G IV -A and/or 5-FU on lung metastases

Experimental group	5-FU		G IV -A		Lung metastases	
	Dose (mg/kg/day)	Day of injection	Dose (mg/kg/day)	Day of injection	No. of surface	Incidence nodules
Control					31.5 ± 8.7	10/10
G IV -A			20	10,12,14,16,18	15.7 ± 5.1	9/10
5-FU	30	10,12,14,16,18			10.6 ± 4.9 *	7/10
G IV -A + 5-FU	30	10,12,14,16,18	20	10,12,14,16,18	5.3 ± 3.2 * *	4/10

Incidence are given as the ratio of number of mice with metastases/number of mice tested. Values are indicated as means ± S.E. of 10 mice. Significant difference: * $P < 0.05$, as compared with control group (undergoing amputation); * $P < 0.05$, as compared with use of 5-FU or G IV -A alone.

Table 4. Enhancing effects of G IV-A, ATSO, zymosan or dextran on chemiluminescence and induction of C3-positive macrophages

Treatment	Maximum of chemiluminescence	C3-positive fluorescent cells (%)
Dextran	0.05 ± 0.02 (1.0)	18.0 ± 3.5
G IV-A	0.31 ± 0.09 (6.2)*	67.5 ± 9.9 *
ATSO	0.40 ± 0.18 (8.0)*	72.9 ± 7.5 *
Zymosan	0.24 ± 0.15 (4.8)*	65.2 ± 5.7 *

Maximum chemiluminescence and C3-positive fluorescent cells were measured at 2 hours after treatment with 20 mg/kg of G IV-A, ATSO, zymosan and dextran (control). Values indicate the mean ± S.E. of 5 mice. * $P < 0.05$ compared with control group. Number in parentheses shows times values to that of control.

And macrophages have phagocytic action. We demonstrated that G IV-A ATSO and Picibanil markedly increased the phagocytic activity and K-index than that of the control mice. Thus, G IV -A acts as a so-called stimulator of the

reticuloendothelial system (RES).

We also examined the effect of G IV-A on splenic T-lymphocytes subsets. Generally, Thy1.2-positive cells are known as a marker of pan T cells. L3T4-positive cells are regarded as a marker of helper/inducer T cells. Asialo GM1-positive cells are known to be NK cells and activated macrophages. In the group receiving G IV-A, the percentages of Thy1.2-, L3T4- and asialo GM1-positive cells were maintained at higher values than in the tumor-bearing control group (Table 5).

DISCUSSION

Many plant polysaccharides, as biological response modifiers exert suppressive effects against solid tumors in mice, and their action is considered to be a host-mediated immune mechanism. However, these polysaccharides were ineffective against the ascites form and spontaneous tumors. We found that G IV-A showed antitumor effect against EAC

Table 5. Analyses of splenic Thy1.2-positive cells, L3T4-positive cells, Asialo GM1-positive cells and Lyt2-positive cells after i.p. administration with G IV-A

	Percentages of positive cell (%)		
	Normal mice	Tumor-bearing mice	
	Saline	Saline(Control)	G IV -A
Thy1.2-positive cells (pan T cells)	50.2 ± 2.7 (100)	32.2 ± 1.9 (64.1) * [100]	46.9 ± 1.8 (93.4) [145.7] #
L3T4-positive cells (helper/inducer T cells)	29.9 ± 3.0 (100)	20.2 ± 1.8 (67.6) * [100]	28.7 ± 1.9 (96.0) [142.1] #
Asialo GM1-positive cells (NK cells and activated macrophages)	12.1 ± 1.9 (100)	12.3 ± 0.8 (101.7) [100]	15.9 ± 0.9 (131.4) * [129.3] #
Lyt2-positive cells (cytotoxic/suppressor T cells)	12.3 ± 1.4 (100)	8.6 ± 0.7 (69.9) * [100]	10.5 ± 0.6 (85.4) [122.1] #

Values are indicated as means ± S.E. of ten mice. Significant difference: * $P < 0.05$, as compared with saline-treated normal mice; # $P < 0.05$, as compared with saline-treated tumor-bearing mice.

and 3LL to inhibit development of the lung metastasis. GIV-A significantly augmented the binding of C3 cleavage product to peritoneal macrophages and the proportion of C3 positive cells. Since the enhancement of both phagocytosis and binding of C3 cleavage product was caused by administration of GIV-A, the enhanced phagocytosis is probably related to C3 activation. On the other hand, it has been shown that some polysaccharides and zymosan ATSO or GIV-A activates an alternative pathway of the complement system.

In the group receiving GIV-A, the L3T4⁺/Lyt2⁺ ratio showed a tendency to increase, and the Lyt2⁺/Thy1.2⁺ ratio was decreased. These results are likely to suggest a decrease in Lyt2-positive cells, which are regarded as a marker of suppressor T cells. This seems to be favorable for cancer immunotherapy.

Tanaka *et al.*⁽¹⁴⁾ concluded that a novel glycoprotein obtained from *Chlorella vulgaris* strain CK22 (CVS) showed antimetastatic immunopotentiality though T cell activation in Lymphoid organs.

The above data leads us to the speculation that the antitumor action of GIV-A may be correlated with the activation of complement C3, macrophages action, stimulation of RES, and the productive abilities of splenic pan T cells, NK cells and helper/inducer T cells. In summary, it is conceivable that GIV-A has immunostimulating effects and immunomodulatory effects. The results at least indicate that some algal polysaccharides might be a valuable host-mediated preparations in the combination modalities in cancer treatment.

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